#28 12/13/02

PATENT
Docket No.: 57953/1115

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Benfey et al.	Examiner: Stuart Baum
Serial No.	:	09/265,585	
Cnfrm. No.	:	4702	Art Unit: 1638
Filed	:	March 10, 1999	
For	:	SCARECROW GENE, PROMOTER AND USES THEREOF	

DECLARATION OF PHILIP N. BENFEY UNDER 37 C.F.R. § 1.132

U.S. Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

Dear Sir:

I, PHILIP N. BENFEY, pursuant to 37 C.F.R. § 1.132, declare:

1. I received a DEUG degree (the French equivalent to a B.S. degree awarded by colleges and universities of the United States of America) in Biochemistry from the University of Paris VI, Paris, France in 1981, and a Ph.D. degree in Cell and Developmental Biology from Harvard University, Cambridge, Massachusetts in 1986.

2. I am currently a Professor and the Departmental Chair of the Department of Biology at Duke University, Durham, North Carolina.

3. I am an inventor of the above-identified application.

4. I am presenting this declaration to show that transgenic plants overexpressing the *Zea mays SCARECROW* (*ZmSCR*) protein (which is identified in my above patent application as having the amino acid sequence of SEQ ID NO:96) exhibit increased cell division in the root and hypocotyls with a concomitant increase of the hypocotyl gravitropic response.

5. As background to the results presented below in paragraphs 6-8, prior studies conducted under my guidance and direction have shown that the *SCARECROW* (*SCR*) gene in *Arabidopsis thaliana* is required for the asymmetric cell division responsible for ground tissue formation in both root and shoot tissue. In *Arabidopsis* roots, expression of the *SCR* gene was observed in the cortex/endodermis initials, the quiescent center (QC), and the endodermis cell lineage (Di Laurenzio et al., "The *SCARECROW* Gene Regulates An Asymmetric Cell Division That Is Essential for Generating the Radial Organization of the *Arabidopsis* Root," Cell 86: 423-433 (1996) ("Di Laurenzio et al., 1996"); Wysocka-Diller et al., "Molecular Analysis of SCARECROW Function Reveals a Radial Patterning Mechanism Common to Root and Shoot," Development 127:595-603 (2000) ("Wysocka-Diller et al., 2000")). Further analysis of *SCR* expression in the shoot revealed that *SCR* was expressed in the seedling shoot apical meristem (SAM), young leaf primordia, bundle sheath cells of the leaf, and the endodermis/starch sheath of the inflorescence stem (Wysocka-Diller et al., 2000). Correlation of the *SCR* expression pattern with radial pattern defects in both shoot and root provides further evidence that *SCR* is needed for the formative cell divisions that give rise to the ground tissue layers. In addition, a detailed study of *SCR* expression during embryogenesis showed that it was consistently expressed in each ground tissue cell before longitudinal division and in the inner daughter cell after division (Wysocka-Diller et al., 2000).

6. Based on sequence similarity and expression pattern in roots, we previously suggested that *ZmSCR* is the likely maize ortholog of the *SCR* gene found in *Arabidopsis* (Lim et al., "Molecular Analysis of the *SCARECROW* Gene in Maize Reveals a Common Basis for Radial Patterning in Diverse Meristems," The Plant Cell, 12:1307-1318 (2000) ("Lim et al., 2000")). In subsequent work, we demonstrated the conservation of *ZmSCR* function by its ability to complement the *Arabidopsis scr* mutant. In addition, we transformed *scr* mutants (i.e., *Arabidopsis* plants exhibiting radial pattern defects in their roots and shoots) with the *ZmSCR* gene under the control of the native *SCR* promoter to see if *ZmSCR* is functionally homologous to *SCR*. As a result, *ZmSCR* was able to rescue the radial pattern defects of the *scr* mutants, suggesting that *ZmSCR* is the functional ortholog of *SCR*. The experimental methods and pertinent results are described in paragraphs 7-8 below.

7. A binary plasmid (named *pSCR::ZmSCR*) containing the open reading frame (ORF) of *ZmSCR* under the control of the native 2.5-kb *Arabidopsis SCR* promoter was generated. The *pSCR::ZmSCR* plasmid was made by placing the full-length cDNA of *ZmSCR* immediately after the 2.5-kb region upstream of the *SCR* translational start site in pSCRHYG (provided by Keji Nakajima). It was shown that the 2.5-kb *SCR* promoter region was sufficient to complement *scr* mutant phenotypes, and the promoter region was able to reflect its native gene expression pattern, when used to drive marker gene expression (Malamy et al., "Analysis of *SCARECROW* Expression Using a Rapid System for Assessing Transgene Expression in *Arabidopsis* Roots," Plant J. 12:957-963 (1997) ("Malamy et al., 1997"); Wysocka-Diller et al., 2000; Nakajima et al., "Intercellular Movement of the Putative Transcription Factor SHR in Root Patterning," Nature 413:307-311 (2001) ("Nakajima et al., 2001")). The resulting plasmid (*pSCR::ZmSCR*) was used to transform *Arabidopsis scr* mutants by the *Agrobacterium*-mediated, floral-dipping method (Clough et al., "Floral Dip: A Simplified Method for *Agrobacterium*-Mediated Transformation of *Arabidopsis thaliana*," Plant J. 16:735-743 (1998) ("Clough et al., 1998")). Seeds from the transformed plants were harvested and plated on medium containing hygromycin (15 mg/ml) to select transgenic plants harboring *pSCR::ZmSCR*. To confirm the genetic background of the transgenic plants, polymerase chain reaction (PCR) products were amplified using the primers specific for the *Arabidopsis SCR* (wild-type). Subsequently, the amplified products were restriction-enzyme digested to observe polymorphisms between wild-type and *scr* plants. In addition, the primers specific for *ZmSCR* were used to verify the presence of the *pSCR::ZmSCR* construct in PCR analysis on the same DNA samples that were used for genotyping.

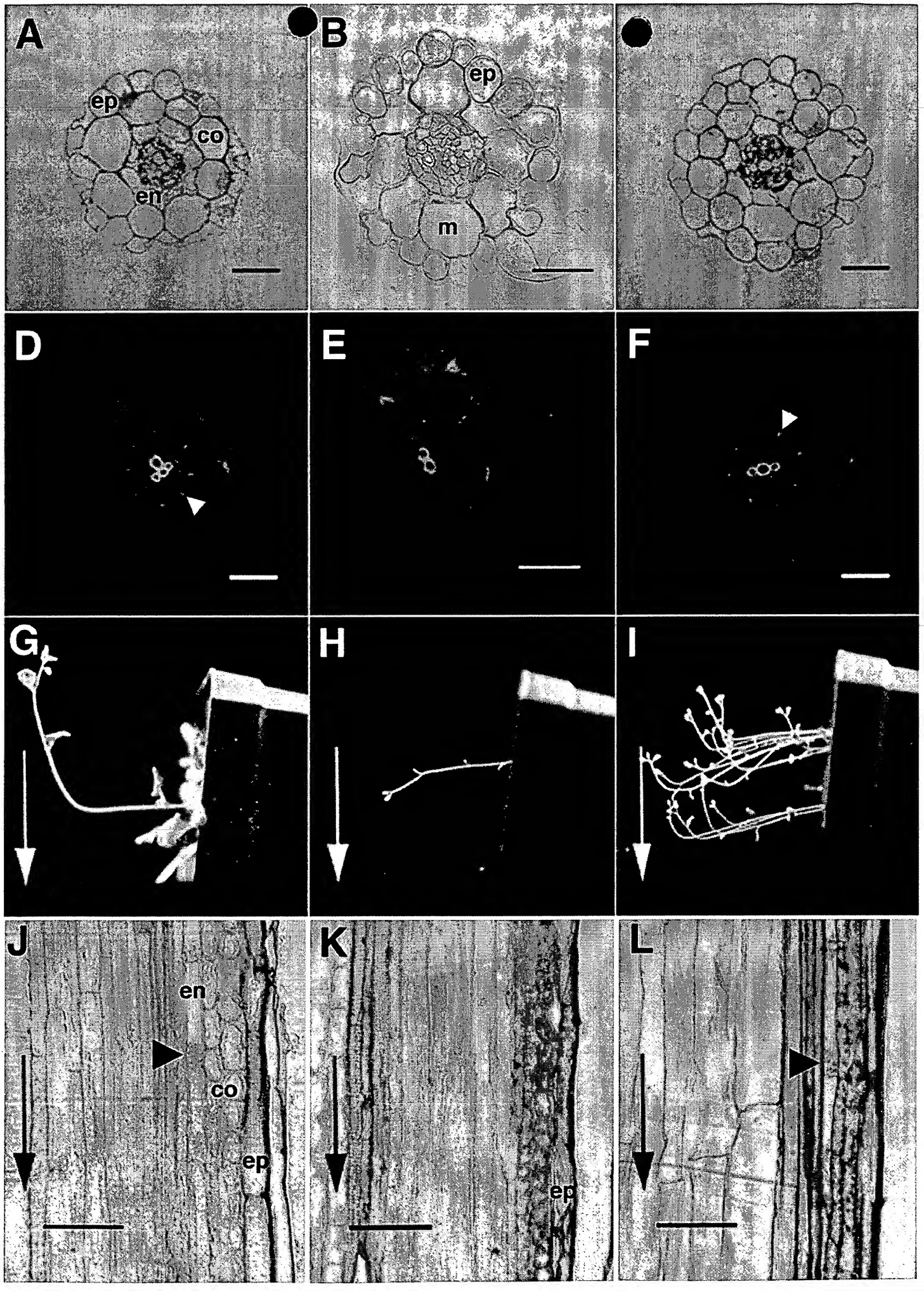
8. Mutations in *scr* result in the lack of one ground tissue layer in the root with the remaining layer exhibiting mixed characteristics of both cortex and endodermis. After selection for transgenic plants resistant to hygromycin, we analyzed the radial organization of the transgenic plants harboring the *pSCR::ZmSCR* construct. As shown in Figures 1A-1L and their accompanying descriptions (attached hereto as **Exhibit A**), transverse sections of the primary root of transgenic plants showed a normal radial organization with a single cortex and an endodermal layer as seen in the wild type plants. Histochemical staining for the Casparian strip, which is differentiated endodermal marker, was applied to characterize the root radial pattern of transgenic plants (Figures 1D to 1F). Our analysis verified the presence of endodermal

characteristics in the inner layer of ground tissue, indicating that *ZmSCR* was able to complement radial defects in *scr* roots (Figure 1F). In addition, the gravitropic response of *scr* mutant inflorescence stems was also restored (Figure 1I). These results show: (1) *ZmSCR*, the maize *SCR*, is functionally homologous to *SCR* in regulating the ground tissue patterning in root and shoot tissue and (2) plants transformed with this gene exhibit increased cell division in root and hypocotyl tissue, resulting in transgenic plants having thicker roots, straighter shoots, and less susceptibility to lodging than non-transgenic plants.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that theses statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Dec. 2, 2002

Philip N. Benfey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Figures 1A-1L. Complementation of *scr* with *ZmSCR*. Figures 1A to 1C shows transverse sections of primary roots. Figure 1A: Wild-type. Figure 1B: *scr*. Figure 1C: *scr* transformed with *pSCR::ZmSCR*. Note the restoration of two ground tissue layers. Figures 1D to 1F show the Casparian strip staining of transverse sections of primary roots. Figure 1D: Wild-type. Figure 1E: *scr*. Figure 1F: *scr* transformed with *pSCR::ZmSCR*. The white arrowheads point out the Casparian strip staining in the endodermis, which indicates the restoration of endodermal characteristics in the inner ground tissue layer. Figures 1G to 1I show the gravitropic response of inflorescence stems. Figure 1G: Wild-type. Figure 1H: *scr*. Figure 1I: *scr* transformed with *pSCR::ZmSCR*. Figures 1J to 1L show longitudinal sections through inflorescence stems. Figure 1J: Wild-type. Figure 1K: *scr*. Figure 1L: *scr* transformed with *pSCR::ZmSCR*. The black arrowheads indicate the amyloplasts that sediment in response to gravity. The direction of gravity is shown by the arrows. Abbreviations: co, cortex; en, endodermis; ep, epidermis; m, mutant cell layer. The bars in Figures 1A to 1L = 50 _m.